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Detection of Antibiotic Residues in Bovine Milk

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DETECTION OF ANTIBIOTIC RESIDUES IN BOVINE MILK

BY

MARK EDWARD JOHNSON

A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science, Major in
Dairy Science, South Dakota
State University

1976

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DETECTION OF ANTIBIOTIC RESIDUES IN BOVINE MILK

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis/Adviser

Date

Head, Dairy Science Department

Date

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INTRODUCTION

Under the Food, Drug, and Cosmetic Act antibiotics are considered as adulterants in milk and milk products. Cultured dairy products cannot be made with milk containing small concentrations of antibiotics. Also, minute quantities of antibiotics cause allergic reactions in some people. Penicillin is the most common antibiotic to which people are allergic, and penicillin is also the most commonly used antibiotic in the treatment of infections in bovines.

Antibiotics, whether injected intravenously or intramuscularly, or infused into the udder of the cow, can be secreted into the milk. It is for this reason that withdrawal times for milk from cows treated with antibiotic preparations have been determined. The withdrawal time is that period following treatment with antibiotics for the antibiotic residue to be completely removed from the milk from treated cows.

The method recommended by the Food and Drug Administration (FDA) for determination of penicillin concentration in milk is the Sarcina lutea cylinder plate method. Although penicillin is not the only drug used for treatment of infections, it is the antibiotic of choice when checking milk for drug residues. Therefore, tests

for the detection of antibiotic residues in milk are usually rated on their sensitivity to penicillin. The two most commonly used methods for penicillin residues in milk are the Bacillus subtilis disc assay and the Sarcina lutea cylinder plate procedures. The cylinder plate method is more sensitive to penicillin than the disc assay method. Recently an antibiotic test has been developed which is reportedly more sensitive to penicillin than the disc assay or cylinder plate methods. It is marketed under the tradename of Delvotest P.

One objective of this study was to determine whether the withdrawal time for milk from cows treated with penicillin would be increased if the Delvotest P method was used. Also, a comparison was made of relative sensitivities of the Delvotest P, disc assay, and cylinder plate tests for determination of penicillin in milk.

Cows are sometimes treated in their dry period to prevent mastitis infections from developing. The "dry cow" antibiotic preparations have withdrawal times recommended by the manufacturer as do all the drugs used in treatment of infections in bovines. The withdrawal times for antibiotics used in dry cow treatments are determined from the time the cow calves. The manufacturer also recommends that dry cow antibiotic treatments not be used for a certain

period of time prior to the expected calving date to insure that no antibiotics will be secreted longer than the recommended withdrawal time. Therefore, another objective of this study was to determine if antibiotics used in dry cow treatments persisted longer than the recommended withdrawal time in milk if the cows calved earlier than expected.

LITERATURE REVIEW

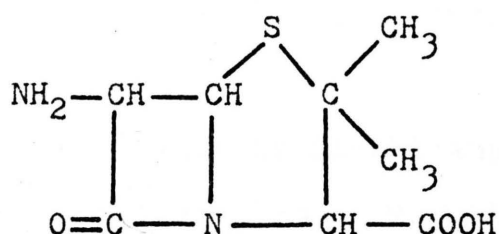
An antibiotic has been defined by Selman Waksman as being an organic compound, produced by one microorganism, that, at great dilution, inhibits the growth of or kills another microorganism or microorganisms (14). Several antibiotics are currently used in the treatment of mastitis and other infections in dairy cows. Penicillins were among the first antibiotics used and are probably the most widely used today (51). A discussion of the structure and mode of action of antibiotics will be limited to penicillin and cloxacillin, a semisynthetic penicillin.

This review will discuss the antibiotic residues in milk following intramuscular injection and intramammary infusion in both the nonlactating (dry) and lactating dairy cow. The development of the cylinder plate, disc assay, and agar diffusion tests for the detection of antibiotic residues in milk will also be discussed.

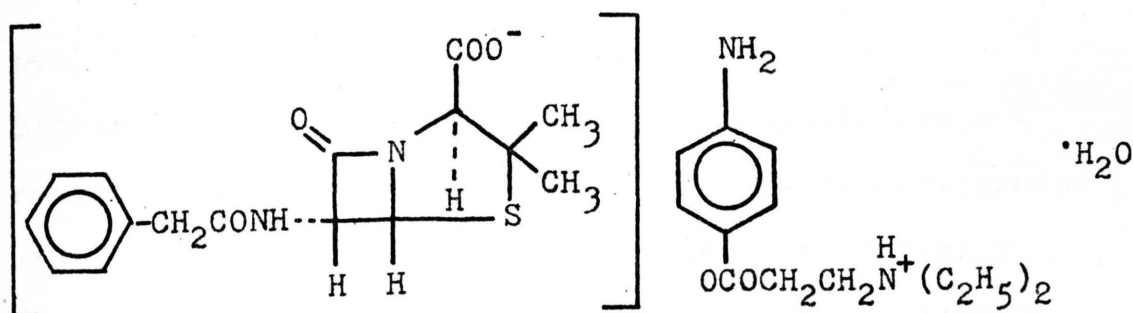
Chemical structure and mode of action of penicillin and cloxacillin

Penicillin is a generic name of a large group of antibiotics produced by strains of the mold Penicillium. These penicillins have a common chemical nucleus and

differ principally in the chemical structure of an acyl side chain attached to this nucleus. The acyl side chains are necessary for penicillin action and are introduced either biologically or chemically. Batchelor et al. (6), isolated and identified the penicillin nucleus as a strong monobasic acid, 6-amino-penicillanic acid, which has the following chemical structure:



Casida (14) described the industrial production of penicillins. He states that the penicillins are inherently unstable molecules when in the free acid form and, therefore, are usually prepared as the much more stable salts or esters. The penicillin used in this study was a procaine salt of benzyl penicillin (penicillin G) called procaine penicillin G and has the following chemical structure:

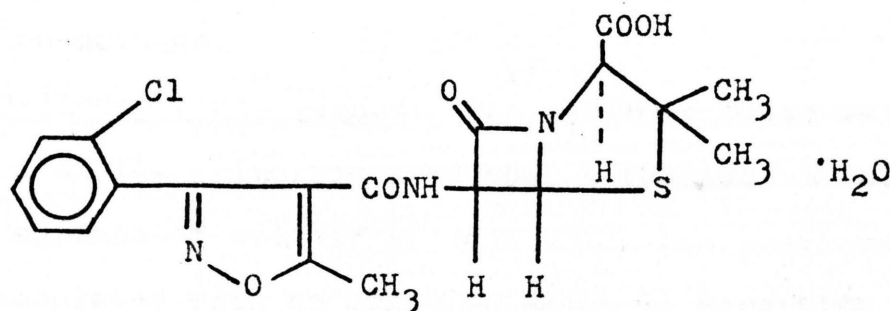


Procaine penicillin G has a relatively low solubility; hence, upon intramuscular injection, it slowly releases the penicillin G and increases the duration of effective blood levels.

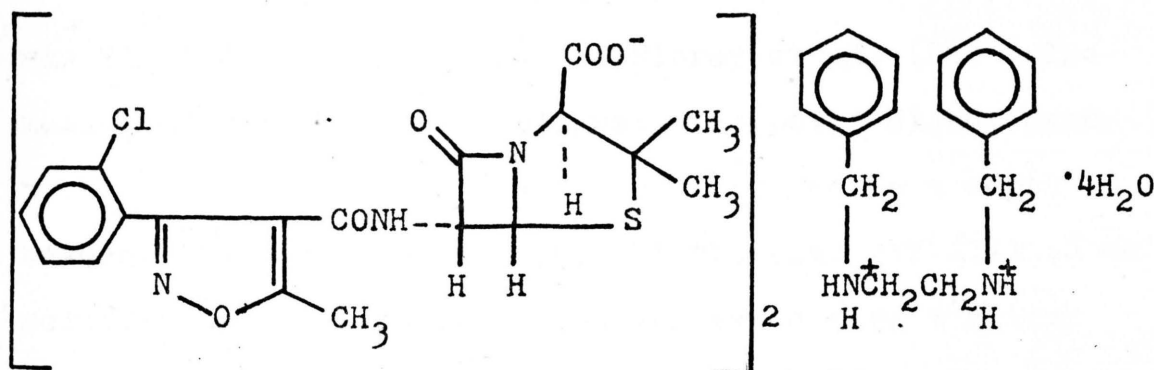
Penicillins act mainly by inhibiting the enzymes concerned with the incorporation of muramic acid into the mucocomplex (peptidoglycan) of the rigid bacterial cell wall (11). Without formation of the rigid, structural layer of the cell wall, the bacterial cell will lyse. Since synthesis of cell wall is carried on only by actively multiplying cells, the antibacterial action is manifested only during active growth (11). Certain bacteria adaptively produce an extracellular enzyme called penicillinase which hydrolyzes penicillin to inactive penicilloic acid. Penicillinase is a major factor concerning the resistance of microorganisms to penicillin. Specific penicillinases will act only on specific

penicillins. Introduction of acyl groups to 6-amino-penicillanic acid leads to penicillinase resistant semisynthetic penicillins. These acyl groups are introduced by reacting appropriate artificially synthesized acyl chlorides with 6-amino-penicillanic acid.

Cloxacillin is one of the hundreds of semisynthetic penicillins. Doyle et al. (17) described cloxacillin as a semisynthetic penicillin resistant to penicillinase, acid stable, and with useful activities against most gram-positive bacteria. Nayler et al. (42) described the chemistry, toxicology, pharmacology, and microbiology of cloxacillin. He stated that cloxacillin is primarily of interest for its activity against penicillin-resistant staphylococci. Nayler et al. (42) reported the structure of cloxacillin as follows:



Cloxacillin, like penicillin, is also an inhibitor of cell wall synthesis and is more stable as a salt. The cloxacillin used in this study was benzathine cloxacillin which has the following chemical structure:



Antibiotic testing methods

Several methods for the detection of antibiotics have been developed and were reviewed by Marth in 1961 (36). This review will be limited to literature on the development of the cylinder plate, disc assay, and agar diffusion methods.

Cylinder plate method: The cylinder plate method is based on the principle that when a cylinder is placed on the surface of solidified agar which has previously been inoculated with an organism which is sensitive to a given antibiotic, and then filled with an antibiotic

solution, the antibiotic will diffuse from the cylinder, inhibit the growth of the test organism, and form a circular zone of inhibition which is directly related in diameter to the concentration of the antibiotic in the cylinder.

The cylinder plate method for the assay of penicillin was first described in 1941 by Florey et al. (21). The method described was as follows: (a) petri dishes were prepared with a nutrient agar layer poured to a depth between the limits of 3-5 mm, (b) the agar was allowed to solidify and the plates were then seeded with the test organism, Staphylococcus aureus, by allowing a broth culture of the organism to flow over the surface of the agar and draining off the excess broth, (c) the plates were then dried for an hour in an incubator at 37 C, (d) cylinders made from short lengths of glass tubing were then placed on the agar and filled with the solution to be tested, (e) the plates were incubated at 37 C for 12-16 hours. They observed that the diameter of the zone of inhibition was only slightly smaller when the cylinder was half-filled than when it was fully filled. They also observed that sometimes the clear zone of inhibition was surrounded by a halo of partial inhibition, which varied from a faint ghost to almost complete inhibition. This halo resulted from lysis of the bacterial cells.

Foster and Woodruff (23) modified the method of Florey (21) by replacing the unwieldy procedure of inoculation with seeding cooled, melted agar before pouring the plates. One-tenth ml of a 24-hour nutrient broth culture of Staphylococcus aureus was used to seed 100 ml of cooled, melted agar. The agar was uniformly measured into each plate by apportioning 13-ml amounts with a pipette. Instead of using cylinders with a sharp, bevelled edge to obtain a seal between the tube and the surface of the agar, unbevelled cylinders were used and were passed momentarily through a flame to warm them slightly. When the heated cylinder is placed on the agar surface it melts the agar locally, which sets again practically immediately, making a good seal.

Foster and Woodruff (24) cited several disadvantages of using Staphylococcus aureus as the sensitive organism in the cylinder plate method. They described a method in which spores of Bacillus subtilis replaced Staphylococcus aureus as the sensitive organism. Schmidt and Moyer (59) also modified the method of Florey (21). They flooded petri dishes containing 22 ml of agar with 3 ml of agar that had been previously inoculated with Staphylococcus aureus. They reported that the size of the zone of inhibition

decreased as the depth of the agar medium in a petri dish increased. When 25 ml of medium per plate was used, the edge of the zone was distinct and clear. However, if only 15 ml were used, the edge of the zone became indistinct.

The most clear cut zone was obtained with the agar medium at pH 6.0 (59). Penicillin is destroyed in a medium of very low pH, however it has been well established that penicillin is more active against susceptible bacteria in a slightly acid than in an alkaline environment. As the pH was increased, the edge of the zone became more indistinct (59). When the inoculum was too heavy, the zones of inhibition were not uniform and consistent. If the inoculum was too light, the zones of inhibition were very large and the edges were ill-defined. Schmidt and Moyer (59) also reported that a prolonged incubation or drying period reduced the size of the zone of inhibition. Abnormally large zones of inhibition were obtained by refrigerating the completed plates an hour or two before incubation (59). Refrigeration retards bacterial growth and permits a longer time for the diffusion of the penicillin. The size of the circle of inhibition is, in part, dependent on the time allowed for diffusion before the test organism produces a substantial amount of growth.

The method of Schmidt and Moyer (59) was modified by Beadle et al. (7) who suggested the use of 14.0 by 29.3 cm rectangular glass dishes instead of petri plates. A guide was used to drop the cylinders onto inoculated culture plates. The methods of Schmidt and Moyer (59) and Beadle et al. (7) were applied to milk for detection of penicillin by Thorp (67). Meeves and Milosevic (39) observed that clear cut zones of inhibition were produced if the test organism was spread over the surface of the agar medium rather than mixed with it. Raw milk, which contained no penicillin, sometimes inhibited Staphylococcus aureus. The presence of Pseudomonas pyocaneum or Bacterium linens in raw milk also inhibited Staphylococcus aureus (39). Because of the problems of using Staphylococcus aureus as the test organism (24, 39), Juncher et al. (31) suggested the use of Sarcina lutea as the sensitive organism for the detection of penicillin. Sarcina lutea was also found to be more sensitive to penicillin than Staphylococcus aureus.

The Food and Drug Administration (FDA) adopted the cylinder plate method and Sarcina lutea as the test organism for penicillin detection in raw milk. The FDA uses a modified method of Schmidt and Moyer (59). The method of the FDA (13) includes the following: (a) 10 ml

of agar medium are poured into the petri plate, (b) 4 ml of agar seeded with a previously standardized suspension of Sarcina lutea (ATCC 9341) are spread over the surface of the hardened base layer, (c) cylinders are put in place and solutions added, (d) the plates are incubated at 32 C for 16-18 hours.

Disc assay method: Foster and Woodruff (23)

revealed a novel simplification of the cylinder plate method developed by Vincent, Vincent, and Dowdy in 1943. Instead of using cylinders, discs of filter paper saturated with penicillin samples were used to produce the zones of inhibition. Where the penicillin diffused out into the agar, growth of the test organism was inhibited and a circular, clear zone resulted. Using a modified B. subtilis cylinder plate method of Foster and Woodruff (24), Welsh et al. (72) assayed milk samples from cows following parenteral administration of penicillin. They prepared plates by pouring 3 ml of agar medium seeded with spores of B. subtilis into each plate. Filter paper discs (7 mm in diameter) were placed on the plates. A .03-ml sample of the milk was added to each disc. The completed plates were incubated at 39 C. Results were read after four hours. Loo et al. (33) developed a paper disc assay method for assaying streptomycin and described the

advantages of using 12.7-mm filter paper discs instead of glass cylinders.

Silverman and Kosikowski (62) studied the disc assay method and used 7-mm filter paper discs in addition to 12.7-mm filter paper discs. The 7-mm filter paper discs were saturated with the milk sample to be tested by holding the disc with tweezers and applying the milk with a micro pipette. The 12.7-mm filter paper discs were impregnated with the milk sample by simply touching an edge of the disc in the milk until the entire disc was saturated by capillary action. Using the 7-mm filter paper discs they were able to detect penicillin in milk in concentrations as low as .1 unit per ml. They reported that the use of the 7-mm disc procedure was restricted to penicillin. The milk to be tested was always heated to 82 C for five minutes to destroy naturally occurring inhibitory substances (71, 73, 74). They also described a method using penicillinase discs to specifically identify penicillin as the inhibitory substance.

Cerny and Morris (15) developed a disc assay test in which two 12.7-mm filter paper discs, placed one on top of the other on the agar surface, were used to introduce the sample to the plate. The double discs absorbed about

ten times as much milk as a single 7-mm disc. They also suggested the use of 6 ml of seeded agar in a flat bottomed dish and incubation of completed plates at 37 C for eight hours or overnight (16-18 hours) at room temperature. The double disc method was found to be sensitive and precise to concentrations as low as .01 unit of penicillin per ml of milk while the 7-mm disc procedure lost precision below .1 unit per ml. Siino et al. (61) studied the sensitivity of the disc assay method as affected by the discs. They found that a single 12.7-mm disc was as sensitive for detecting penicillin as the double 12.7-mm disc. The 12.7-mm discs were more sensitive than either single or double 7-mm discs, and double 7-mm discs showed little advantage over the single 7-mm disc. The larger size of the 12.7-mm disc made them easier to handle and they absorbed milk uniformly while absorbency by the 7-mm discs was not uniform. On the basis of greatest convenience and highest sensitivity, they concluded that the single 12.7-mm disc was the most suitable for use in the disc assay method.

Arret and Kirshbaum (5) developed a rapid disc assay method for detecting a minimum concentration of about .05 units of penicillin per ml of milk in 2.5 hours. Johns (29) criticized their method as being less simple,

less reliable, and less sensitive than claimed. Marth et al. (37) modified the method of Arret and Kirshbaum (5) and developed a rapid disc assay test that could detect a minimum concentration of about .03 units of penicillin per ml of milk in 3-4 hours when incubated at 37 C.

Studies on the agar used in the disc assay test (15, 30, 37) showed that the greatest sensitivity was obtained with 6 ml penassay seed agar, (Antibiotic Medium No. 1) (4). Marth et al. (37) showed that for best results the inoculated agar should contain approximately $.1 - 1.0 \times 10^6$ spores of B. subtilis (ATCC 6633) per ml when using the disc assay procedure to detect penicillin residues. The disc assay method, as described in Standard Methods (3), is that of Silverman and Kosikowski (62) with modifications of several workers.

Agar diffusion test: Agar diffusion tests, to detect the presence of antibiotics in liquid samples, are based on the survival characteristics of bacterial spores. Agar lacking all nutrients is seeded with spores of a sensitive organism. Elimination of all nutrients from the agar prevents germination of the spores. When performing the test, nutrients are added along with the liquid sample to the agar medium and both diffuse throughout the agar. Germination and growth of the spores is initiated when the

nutrients are added. However, if the liquid sample contains antibiotics to which the test organism is sensitive, the organism will not grow. Growth or lack of growth of the test organism is detected by visual means.

Kosikowski and Ledford (32) developed the first agar diffusion test or reverse-phase disc assay test for detection of antibiotic residues in milk. They seeded plain saline agar with spores of B. subtilis and pipetted 10 ml of this seeded agar to each petri plate. A special nutrient-packed paper disc, moistened with the milk being tested, was applied to the surface of the agar and the plate was incubated at 37 C for 4-6 hours. After incubation, the plates were visually examined for bacterial growth. A growth area next to a disc indicated normal milk, while a clear area of no growth around a disc indicated the presence of antibiotics. Utilizing microscopic examination, test results could be obtained in 2.5-3 hours.

The rapid growth of B. stearothermophilus at elevated temperatures and its high sensitivity to penicillin and other antibiotics (28) indicated its potential usefulness as a test organism for detection of antibiotics. Using the same principle as Kosikowski and Ledford (32), van Os et al. (70) developed an agar

diffusion test with Bacillus stearothermophilus var. calidolactis as the sensitive organism. Tablets containing nutrients and a pH indicator, bromocresol purple, were added to ampules containing spores of B. stearothermophilus var. calidolactis. After adding .1 ml of the milk sample, the ampule was incubated for 2.5 hours in a water bath at 63-66 C. The nutrients, pH indicator, and antibiotics, if present in the milk, diffuse into the agar medium. The bromocresol purple changes the color of the agar to a purple color. As the Bacillus organism grows, it lowers the pH of the medium and the pH indicator bromocresol purple will change the color of the agar to yellow. If antibiotics are present in a sufficient concentration to inhibit the growth of the Bacillus organism, the organism will not grow, the pH of the medium will not decrease, and the agar medium will remain a purple color. Materials for this method are now available in kits which are marketed by Gist-Brocades nv, P. O. Box 1, Delft, Holland, under the name Delvotest P, and distributed in the United States by Enzyme Development Corporation, 2 Penn Plaza, New York, N. Y.

Antibiotic residues in milk

Excellent reviews of the literature concerning antibiotic residues in milk following treatment of cows with antibiotics are given by Albright et al. (2), Marth (35), and Marth and Ellickson (38). This review will be concerned with literature on the penicillin and cloxacillin residues in milk from cows receiving dry cow treatments, and with penicillin residues in milk from lactating cows following intramuscular injection and intramammary infusion.

Dry cow treatment: Literature reporting antibiotics in milk from lactating cows that have received a dry cow antibiotic treatment is scanty. A few reports have been published on the persistence of antibiotics in the dry udder. Pearson (44) infused procaine penicillin in an oil base into the dry udder. The antibiotic level declined very rapidly in the dry udder but persisted at very low levels for up to 12 days. Uvarov (68) in a similar trial found low levels of penicillin for 21 days. Retention of penicillin in dry udder secretions showed considerable variations and appeared to be greatest in cows that had been dry the longest. Antibiotics were found in the milk of a few cows after calving but again the retention was scattered. Smith et al. (63) infused .5 g and 1.0 g of benzathine cloxacillin in a mineral-oil

base into the udders of six cows. Cloxacillin was found in the dry udder secretions for up to three weeks. When .2 g of benzathine cloxacillin was infused in the udder of a dry cow the antibiotic was found in the dry udder secretions for less than one week (63). These findings were confirmed by Schmid (58).

Smith et al. (64) infused 1 and 5 million units of penicillin G in a quick release base into quarters of cows after the last milking of lactation. Penicillin persisted in infused quarters for about four days. Pugh et al. (46) infused all quarters of 150 cows with a single infusion of 1 g procaine penicillin G and .5 g dihydrostreptomycin in a long acting oil base. Penicillin levels in dry cow secretions persisted for at least three weeks after infusion. Dihydrostreptomycin was present for four to five weeks. However, all milk samples taken more than four days after calving showed no detectable antibiotic residue. Of 412 samples taken within four days of calving, only two samples were found to have detectable antibiotic residues. The dry period of the 150 cows treated ranged from 14-245 days.

Ziv et al. (79) studied the retention of antibiotics in dry-udder secretions after infusion of several commercially available dry cow antibiotic preparations. They concluded that the persistence of antibiotics in

the secretions of the dry udder is governed by their rate of absorption from the udder into the general blood circulation. They also indicated that the absorption rate of different antibiotics was independent of the dose or the nature of the vehicle used to carry the antibiotic. Ziv et al. (78) studied the rates of decline in the concentrations of several antibiotics in the secretion of the dry udder after cows were infused on the last day of lactation. They found that, in general, the rate of disappearance of the antibiotics from the secretions was exponentially linear and to some extent independent of the dose. They suggested that drugs were absorbed from the udder by passive diffusion.

Rasmussen (50) stated that in the non-lactating udder the antibiotic concentration will decrease because of hydrolysis in the udder secretion, and by diffusion and absorption into the body and blood circulation of the cow. Funke (25) showed that diffusion through the udder in the dry period was similar to that of the normal lactating gland. Penetration of antibiotic into the tissue appeared to be improved when the cow was not lactating.

Intramuscular injection: Watts and McLeod (71) injected up to 1×10^6 Oxford units of calcium penicillin intramuscularly into normal heifers and were unable to detect penicillin in milk. They concluded that the lack

of penicillin in the milk was due to destruction of penicillin by the gland or selective activity on the part of the secretory epithelium. Welsh et al. (72) thought that if penicillin blood concentrations were sufficiently high and persistent, penicillin could be demonstrated in milk. They administered 17,250,000 units of sodium penicillin G subcutaneously to a normal 628 kg cow in four doses given at six hour intervals. Using the B. subtilis cup-plate method of Foster and Woodruff(24) they were able to detect small quantities of penicillin in milk for up to 30 hours. They concluded that there is diffusion of penicillin from blood to milk.

Edwards and Haskins (18) using the assay method of Florey et al. (21) detected penicillin in milk of a cow up to 24 hours after intramuscular injection of 11 mg of penicillin per kg of body weight. Sadek (55) found that doses of 5,000 units of potassium penicillin G per .45 kg of body weight resulted in small concentrations in the milk for 24 hours. Similar results were obtained by Wright and Harold (75). Cannon et al. (12) administered 3,000 units of potassium penicillin G intramuscularly and found penicillin in samples for up to 72 hours. Blobel and Burch (9) injected crystalline procaine penicillin G into muscle tissue of 24 cows at dosages of 3,000 and 6,000 units per .45 kg of body weight, respectively. At both

dosage levels measurable amounts of penicillin did not persist in the milk of any of the 24 cows 60 hours after injection. In similar studies Wright and Harold (75) and Vaid et al. (69) found penicillin residues in milk for up to 60 hours. After a single intramuscular injection of 4,000 units of penicillin per .45 kg of body weight, Prouty (45) found penicillin in milk for up to 48 hours. Similar results were obtained by Olson and Krawczyk (47) and Schipper et al. (57) who injected a single dose of 3×10^6 units into lactating cows. Cosgrove and Etgen (16) showed that of 25 cows given intramuscular penicillin injections in amounts varying from 3×10^6 to 6×10^6 units per cow, four did not show any antibiotic residue in the milk.

It has been observed that penicillin residues persisted in the milk of low producing cows for a longer period than in the milk of high producing cows (9, 47, 75). Notable breed differences in retention of penicillin were not observed either. Penicillin in an oil base has been given intramuscularly with withdrawal times up to 5.5 days (9, 57, 75). However, Albright et al. (1) did not detect penicillin in milk samples after injecting 6×10^6 units of penicillin in oil into the muscle of several healthy and mastitic cows. Penicillin in oil for intramuscular injection is currently not being used

because of the prolonged duration in tissue of treated animals.

Intramammary infusion: When penicillin was first used in mastitis therapy it was administered by intramammary infusion. Penicillin was infused into an infected quarter in aqueous vehicles. Schalm and Casselberry (56), Thorp et al. (67), and Spencer et al. (65) found that a significant quantity of penicillin was still present 24 hours after infusion. It was observed that the volume of milk excreted by the quarter receiving treatment had a greater influence on the efficacy of penicillin infusions than the extent of tissue damage (56). There was a disadvantage with using aqueous vehicles in that several repeated doses were necessary to cure a case of mastitis. It was suggested that if a vehicle other than water could be devised to carry penicillin into the udder a more effective dose could be retained in the udder for extended periods of time. Foley et al. (22) prepared two water-in-oil bases, in which penicillin was dissolved, for udder infusion. Penicillin could be detected in milk samples 72 hours after infusion of 1×10^5 units of penicillin. They concluded that the persistence of penicillin was longer with water-in-oil preparations than with aqueous carriers due to the greater dispersion of the water-in-oil base into the upper portion of the gland

coupled with a slower excretion of the penicillin from the water-in-oil vehicle.

The conclusions of Foley et al. (22) are contrary to a report by Hueber et al. (27) who stated that aqueous infusions are superior to ointments or oil suspensions in therapy, due to their greater spreading and penetration within the udder. Edwards and Haskins (18) confirmed the findings of Foley et al. (22) and also concluded that the concentration of penicillin in the milk was inversely related to the milk yield. Blobel (8) observed the transfer of penicillin from the treated to the untreated quarters when penicillin in a water-in-oil emulsion vehicle was infused into quarters of lactating cows at dosages of 1×10^5 and 3×10^5 units of penicillin per quarter. When 1×10^5 units were infused, penicillin concentrations in milk samples from the untreated quarters ranged from .005-.010 units per ml. When 3×10^5 units of penicillin were infused, penicillin concentrations in milk from untreated quarters ranged from .005-.060 units per ml. Penicillin persisted in the treated quarters for up to 120 hours and in untreated quarters for up to 36 hours. Blobel (8), using the cylinder plate method, also reported that the concentrations of penicillin transferred to the untreated quarters would hardly be detectable with less sensitive assay methods. He observed that generally

penicillin persisted in the milk of low producers at higher concentrations for longer periods than in the milk of high producers.

Cosgrove and Etgen (16) observed antibiotic crossover in four of 33 animals given intramammary infusions. One had residues for one milking, two for two milkings, and one for three milkings. Penicillin in treated quarters persisted from 4-15 milkings. Evans and Stern (19) treated cows with the maximum prescribed separate dosages of aqueous base and oil base procaine penicillin G over a three day period. They observed that penicillin was transferred to untreated quarters in similar amounts in both treatments. The penicillin concentration in untreated and treated quarters was greatest in low producing animals. Ormiston et al. (49) detected a greater degree of crossover in cows receiving aqueous suspensions of penicillin than in cows receiving penicillin in an oil suspension. The milk of two of three mastitic cows showed no transfer of penicillin from treated to untreated quarters when the infected quarter was infused with either oil or aqueous preparations. They reported that the zones of inhibition of the milk from untreated quarters were quite small and questionable in some cases. They used the method of Arret and Kirshbaum (5) to assay milk samples.

Antibiotic residues in milk from treated to untreated quarters in dairy cattle has been substantiated by several other workers (1, 10, 20, 26, 34, 52, 54, 60). Brown et al. (10) suggested that crossover occurs via the venous system and secretory tissues rather than by direct infusion through udder tissue. Rollins et al. (52) and Ziv et al. (76) indicated that some mechanism other than direct diffusion from the bloodstream could be involved. Another study has also shown that the bloodstream was not the sole mode of transfer of penicillin to the noninfused quarters (26). Miller et al. (41) studied the drug movement of several weak organic acids when infused into the quarters of healthy cows and concluded that the passage of these compounds into bovine milk could be explained by passive diffusion.

Different antibiotic preparations have different withdrawal times. Each commercial antibiotic preparation has its own withdrawal time that must be approved by the FDA. Therefore a wide range of withdrawal times of several intramammary infusion products have been cited (8, 10, 16, 20, 34, 40, 52, 60).

MATERIALS AND METHODS

This investigation was actually two separate studies. The first study involved the persistence of antibiotic residues in milk from cows that received an infusion of a dry cow antibiotic preparation prior to calving. The second study placed greater emphasis on a comparison of the sensitivity of three tests in the detection of antibiotic residues in milk from cows that received intramuscular injections and intramammary infusions. Incidental to checking for minute quantities of antibiotic residues, persistence was also measured in this study.

Dry cow treatment

Nineteen dry Holstein dairy cows from the SDSU dairy herd were treated, via intramammary infusion of two quarters, with one of two commercial dry cow antibiotic preparations, designated here as antibiotic preparations A and B. The cows were treated approximately twelve days prior to the expected parturition date. The treatment of the animals was by the SDSU dairy herdsman.

Each quarter treated with an antibiotic preparation was infused once with one 10-ml plastet of the antibiotic preparation. The two quarters not treated with an

antibiotic preparation were each given a placebo of 10-ml distilled, sterile water. Two quarters were treated with an antibiotic preparation and two quarters were treated with a placebo to determine if antibiotics would cross over from treated to nontreated quarters. Cows were treated according to the design in Table 1.

Each 10-ml disposable polyethylene plastet of antibiotic preparation A contained the equivalent of 500 mg of benzathine cloxacillin in 7.5 g of suitable base. Each 10-ml disposable plastet of antibiotic preparation B contained 1×10^6 units of procaine penicillin G, micronized, and 1 g of dihydrostreptomycin sulfate, micronized, in an extended action base consisting of 1% w/v hydrogenated peanut oil, 3% w/v aluminum monostearate, and peanut oil USP, q.s.

Milk samples from all four quarters were collected beginning with the first milking post-calving. Samples were collected from all successive milkings until antibiotic tests were negative for two consecutive milkings.

Intramuscular and intramammary treatment

Six lactating Holstein dairy cows were treated, by intramuscular injection and intramammary infusion, with a commercial antibiotic preparation. Treatment by

Table 1. Experimental design for dry cow treatment.

No. of cows	Antibiotic preparation ^a	Quarters treated ^b	
3	A	LF	RR
2	B	LF	RR
2	A	LF	LR
4	B	LF	LR
2	A	RF	RR
2	B	RF	RR
1	A	RF	LR
2	B	RF	LR
1	B	LR	RR

^aAntibiotic preparations described in text.

^bLF=left front, LR=left rear, RF=right front, RR=right rear.

intramuscular injection consisted of one 10-ml injection into the neck or shoulder muscle each day for five consecutive days. Treatment by intramammary infusion consisted of infusing the contents of one 25-ml disposable syringe into the left rear quarter each day for three consecutive days. Cows were treated after the morning milking by the SDSU herdsman. The treatment schedule was established so that each treatment would consist of treating each cow with the maximum dosage for the maximum number of consecutive treatments that were recommended by the manufacturer of the antibiotic preparation. Each of the six cows were treated twice with each type of treatment over a twelve consecutive week period. Treatment of the six animals was performed randomly as given in Table 2.

The formulation for the intramammary infusion contained the following ingredients per 25-ml disposable syringe: Procaine penicillin G, 1×10^5 units; neomycin sulfate equivalent to neomycin base, 100 mg; sulfamethazine, 1500 mg; and hydrocortisone acetate, 20 mg in a homogenized system with a specially prepared base containing 50 mg chlorobutanol added as preservative. Each 10-ml dose of the antibiotic preparation used for intramuscular injection consisted

Table 2. Experimental design for treatment of cows by intramammary infusion and intramuscular injection.

Week	Treatment		Rep.	Set
	A ^a	B ^b		
1	2845 ^c	2955	1	1
2	3287	3136	1	1
3	3092	3167	1	1
4	2955	2845	1	2
5	3136	3287	1	2
6	3167	3092	1	2
7	2955	3136	2	1
8	2845	3167	2	1
9	3287	3092	2	1
10	3136	2955	2	2
11	3167	2845	2	2
12	3092	3287	2	2

^aIntramuscular injection.

^bIntramammary infusion.

^cCow identification number.

of 3×10^6 units of procaine penicillin G with .13% methylparaben and .02% propylparaben as preservatives, .5% lecithin, 1% sodium citrate, .5% polyvinylpyrrolidone, not more than .01% sodium formaldehyde sulfoxylate, and .075% sodium carboxymethylcellulose.

Milk samples from all four quarters of each cow were collected from each milking after the initial treatment. Samples were collected from all successive milkings until two consecutive samples did not show the presence of antibiotics.

Assay procedures

Sample testing: All milk samples from cows treated with a dry cow antibiotic preparation were tested for the presence of antibiotics by using the Sarcina lutea cylinder plate method (13, 53). Milk samples from cows treated with either intramuscular injection or intramammary infusion were tested for antibiotic residues using the Bacillus subtilis overnight disc assay method (3) and the Sarcina lutea cylinder plate method (13, 53). The last positive sample and succeeding negative milk samples tested by the cylinder plate method were also tested for antibiotic residues using the Delvotest P until two succeeding samples were negative. All milk samples were tested within 48 hours of being taken. Samples were

stored at 4 C until analyzed.

To determine if the zones of inhibition resulted from penicillin, penicillinase-impregnated discs were placed near the disc or cylinder with the test sample (3). If the zone of inhibition around the test sample was decreased near the penicillinase-impregnated disc, penicillin was present. If no change in the zone of inhibition around the test sample was observed the inhibitory substance was not penicillin. The inhibitory substance in milk samples from cows given intramammary or intramuscular treatments was confirmed to be penicillin. The practice of using the penicillinase-impregnated discs on all samples from cows given these two treatments was thus discontinued after the first two weeks. Samples taken after the last intramammary infusion, however, were checked since the infusion product contained antibiotics other than penicillin. All milk samples from cows given the penicillin containing dry cow treatment were also checked for the presence of penicillin with the penicillinase-impregnated discs.

Disc assay method: The overnight disc assay procedure was applied as outlined in Standard Methods for the Examination of Dairy Products (3). Spore suspensions of Bacillus subtilis (ATCC 6633) were

obtained from Difco Laboratories. The 1-ml vials of the spore suspension were diluted 1:10 with potassium dihydrogen phosphate buffer, pH 7.2, (34 g of KH_2PO_4 in 500 ml of distilled water, adjusted to pH 7.2 with 1 N NaOH solution, and made up to 1 liter with distilled water) (3).

Plates were prepared by inoculating 1 ml of the 1:10 dilution of spore suspension into 100 ml of Antibiotic Medium No. 1 at a temperature of 55 C. Six-ml portions of the seeded agar were then pipetted into 100-mm glass, flat bottomed petri dishes. After the agar had solidified, the plates were stored in a 4 C refrigerator until they were used. No plates were used immediately after preparation but were stored at least overnight at refrigeration temperatures before using.

With a cleaned, flamed forceps the edge of a nonsterile 12.7 mm filter paper disc (S&S, No. 740-E) was touched into a well-mixed sample of milk. The milk was allowed to completely wet the disc. Excess milk was always removed by touching the disc to the side of the sample bottle. The disc was then immediately placed on the agar surface. Care was taken to avoid placing the disc near the center of the petri dish, since oval zones of inhibition resulted if discs were placed near the

dish's center. Three to six discs could be placed on each petri dish depending upon the expected size of the zones of inhibition. Care was taken to avoid overlapping zones. Milk samples on plates with overlapping zones were retested after diluting the samples to a concentration of about .05 units of penicillin per ml of milk. After plating was completed, the plates were inverted and incubated at 32 C for 12-14 hours. After the incubation period, the diameters of the zones of inhibition were measured with a divider to the nearest .5 mm.

Sarcina lutea cylinder plate method: The Sarcina lutea cylinder plate method was applied as outlined by Carter (13) with suggestions by Ronning of the USDA Laboratory in Minneapolis (53).

The test organism used throughout the analysis was derived from Sarcina lutea (ATCC 9341) obtained from the Land-O-Lakes Plant Laboratory in Volga, South Dakota. The original culture was streaked on an agar slant of Antibiotic Medium No. 1, and allowed to incubate for 24 hours at 32 C. The growth was washed from the slant with sterile physiological saline (.85% NaCl) and transferred to a small Roux bottle containing 12 ml of solidified Antibiotic Medium No. 1. The Roux bottle was incubated for 24 hours at 32 C. After incubation the growth was

washed from the agar surface with 100 ml of sterile physiological saline. The entire suspension was poured into a sterile bottle and stored in a refrigerator for no longer than two weeks (13). This stock culture suspension served as the inoculum for the next stock culture prepared, instead of using the slant culture as described by Carter (13). New stock cultures were prepared every ten days.

The contents of test tubes containing 12 ml of sterilized Antibiotic Medium No. 1 were poured into 100-mm, glass, flat-bottomed petri dishes. After the agar had solidified, the petri dishes (with covers off) were dried in a 39 C incubator for 50 minutes (53). The covers were replaced after drying. One hundred and fifty ml of sterilized Antibiotic Medium No. 4 were inoculated with enough stock culture to obtain a concentration in the agar of about 7.5×10^5 organisms per ml. Four ml of the inoculated agar were then added to each plate, making sure that the agar was evenly distributed over the base layer of Antibiotic Medium No. 1. After the agar had solidified, the petri dishes (with covers off) were dried in a 39 C incubator for 25 minutes (53). The covers were replaced after drying and the plates were used immediately.

Five stainless steel cylinders were dropped onto the agar surface of each plate from a height of about 4 mm with tweezers. The cylinders were not flamed before dropping them onto the plate. The outside diameter of the cylinders was 8 mm; the inside diameter was 6mm; and the length was 10 mm. Only five cylinders were dropped on a plate to avoid overlapping zones. Pasteur pipettes were used to fill three cylinders with test sample (53). The other two cylinders on opposite sides of the plate were filled with a standard concentration of penicillin containing .05 units per ml (u/ml). Plates were incubated with covers off in a 32 C incubator for 16-18 hours. The atmosphere in the incubator was kept moist with a sponge set in a pan of water. The moist atmosphere kept the plates from drying excessively (53). After incubation the diameters of the zones of inhibition were measured. The diameters were measured to the nearest .5 mm with a divider.

Agar diffusion method (Delvotest P): The materials for the Delvotest P were obtained from Enzyme Development Corporation, New York, N. Y. The testing method followed was that indicated in instructions supplied by the company with test kit materials. Using a disposable syringe, .1 ml of mixed milk sample was added to an

ampule of clear, solid agar medium, seeded with Bacillus stearothermophilus var. calidolactis. To each ampule (broken at the neck) was added one nutrient-indicator tablet which turned the agar purple. The ampules were placed in a suitably sized beaker with enough water added to the beaker to bring the level of the water at least 13 mm above the level of the agar surface. The beaker was covered with aluminum foil. The ampules thus prepared were incubated in a water bath at 64 C (+2 C). As a control, an ampule tested with antibiotic-free milk was always incubated with the ampules containing test samples. When the agar in the ampule with the antibiotic-free milk turned yellow, indicating a negative test, the incubation of all ampules was stopped. All samples that remained purple were considered positive. A doubtful test was indicated when the agar was neither totally yellow nor totally purple. The purple color had to be at least 4 mm below the agar surface for the test to be considered as doubtful. Incubation time was always between 2.5 hours and 2.75 hours.

Preparation of the standard curves: Standard curves were prepared for only cloxacillin and penicillin. Sodium penicillin G was used as the standard for penicillin. Sodium cloxacillin was used as the standard for benzathine

cloxacillin. A small quantity of the antibiotic under test was accurately weighed and dissolved in 100 ml of 1% phosphate buffer, pH 6.0 \pm 1 (8.0 g monobasic potassium phosphate, 2.0 g dibasic potassium phosphate diluted to 1 liter with distilled water and adjusted to pH with 18 N phosphoric acid or 10 N potassium hydroxide) (4). A portion of the penicillin stock solution was diluted with 1% phosphate buffer to give a concentration of 10 units penicillin per ml.

A portion of the cloxacillin stock solution was diluted with heat treated 2% milk to give a concentration of 10 μ g per ml. The 2% milk used for this dilution and for all other dilutions was steamed for two hours to caramelize the lactose and give the standard solutions a darker color than the samples that were tested. The darker color of the standard solutions made it easier to identify the cylinders with the reference concentration in the cylinder plate test. Figure 1 shows the step by step dilution of the stock penicillin and cloxacillin solutions to give the desired antibiotic concentrations. The various concentrations of the antibiotic standard solutions were tested using the same techniques as with the unknown samples using both the cylinder plate and disc assay methods. Zone diameters were measured and

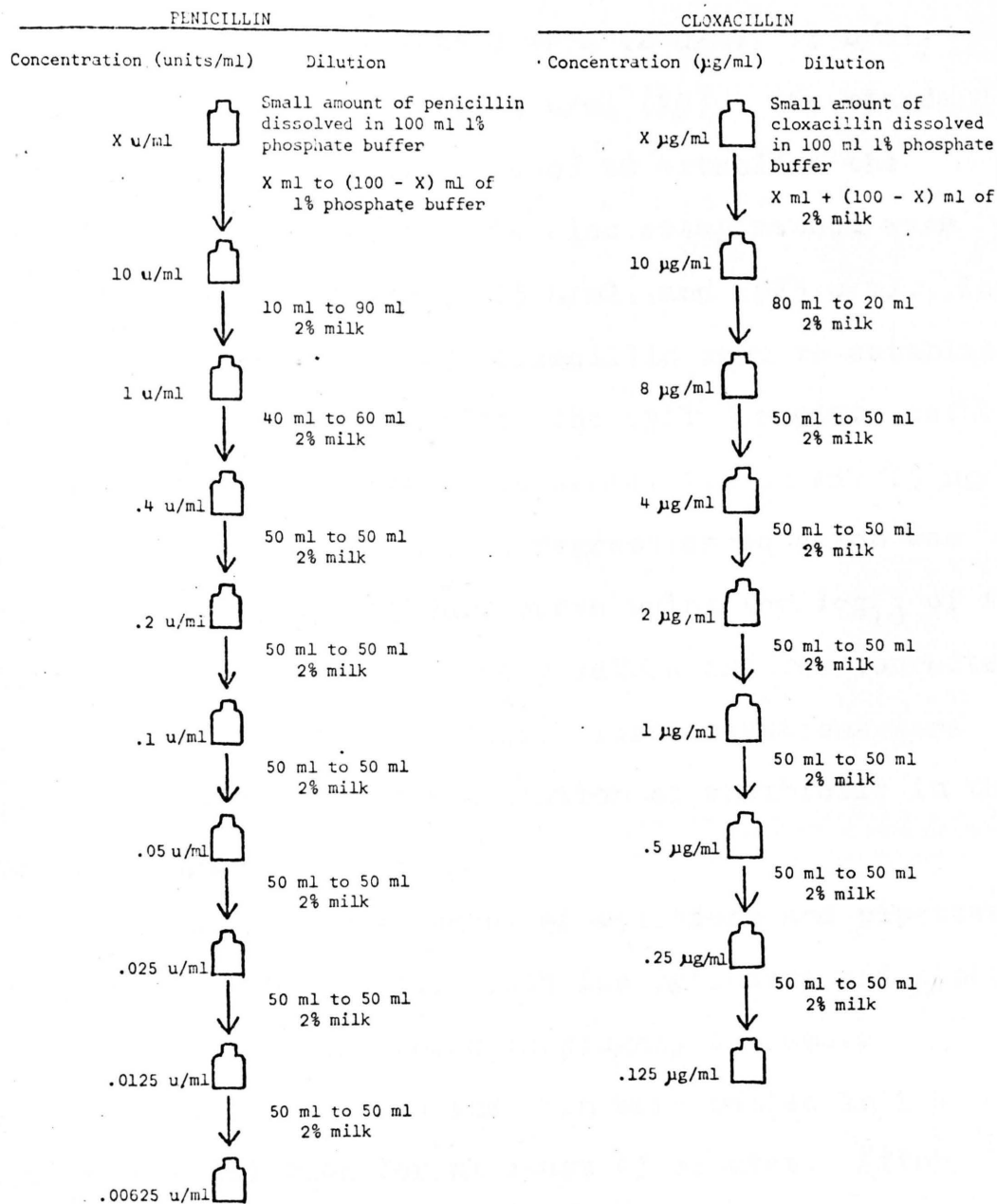


FIG. 1. Preparation of penicillin and cloxacillin standards.

recorded as before. The standard concentrations of penicillin used to establish the standard response line for the cylinder plate method were .2 u/ml, .1 u/ml, .05 u/ml, .025 u/ml, and .0125 u/ml (13). The standard concentrations of penicillin used to establish the standard response line for the disc assay method were .4 u/ml, .2 u/ml, .1 u/ml, .05 u/ml, and .025 u/ml. The standard concentrations of cloxacillin used to establish the standard response line for the cylinder plate method were 8.0 $\mu\text{g/ml}$, 4.0 $\mu\text{g/ml}$, 2.0 $\mu\text{g/ml}$, 1.0 $\mu\text{g/ml}$, .5 $\mu\text{g/ml}$, .25 $\mu\text{g/ml}$, and .125 $\mu\text{g/ml}$. A regression equation was calculated for each standard curve using the \log_{10} of the standard concentration as the y values and the corrected zone diameters as the x values. These equations were used to determine the concentration of antibiotic in the unknown samples.

Due to the large number of cylinders and pipettes used, they were recycled. Both the cylinders and pipettes were first washed or rinsed thoroughly to remove noticeable milk residues and then were boiled in 15% nitric acid solution for at least 45 minutes. After boiling, pipettes and cylinders were rinsed several times with tap water and then with several rinsings of distilled water. After drying both were ready for use.

Statistical analyses: Statistical analyses were conducted on only the data from the study on the comparison of the disc assay, cylinder plate, and Delvotest P methods for detecting penicillin residues in milk. The significance of variations in penicillin titer and withdrawal times were measured by least squares analysis of variance. Mean withdrawal times were compared by Duncan's multiple range test (66).

RESULTS AND DISCUSSION

Antibiotic residues in milk after dry cow treatment

The data of the dry cow experiments are given in Appendix Tables 1 and 2. Cloxacillin was rarely detected in milk from the eight cows treated by intramammary infusion of 500 mg of benzathine cloxacillin during the dry period. After the cows had calved, cloxacillin was detected in the milk of only two cows and then only for one milking. Detectable penicillin residues were observed in the milk from seven of 11 lactating cows that were treated by intramammary infusion of 1×10^6 units of procaine penicillin G during the dry period. However, penicillin residues were not detected in milk samples taken from cows that were treated more than nine days prior to calving. With the exception of two cows, nine cows that were treated with penicillin nine days prior to calving had detectable penicillin residues in the milk from treated quarters after calving. Penicillin crossover from treated to nontreated quarters did occur in one cow (cow 3315), however this cow calved 24 hours after treatment. Due to the extremely short interval between treatment and calving the data from cow 3315 should be considered as being similar to the results obtained after the treatment of a lactating cow.

However, it does emphasize the importance of avoiding treatment of dry cows just prior to parturition to insure that no antibiotic is carried over into the milk.

Ziv et al. (78) stated that in the dry udder the rate of disappearance of seven different antibiotic preparations was exponentially linear. Our results show that in the lactating cow, antibiotic residues were generally not excreted exponentially, but were excreted slowly until complete disappearance (Appendix Table 2).

The minimum concentration of cloxacillin detectable by the Sarcina lutea cylinder plate test used in this study was .25 μ g of cloxacillin per ml of milk. Smith et al. (63) and Ziv et al. (79) detected cloxacillin residues in dry udder secretions of cows for three weeks following the infusion of 500 mg of benzathine cloxacillin. Both groups used the Sarcina lutea cylinder plate method, which detects a minimum concentration of cloxacillin of .25 μ g per ml. The same method was used in this study. In our study cloxacillin was detected in the milk of only two cows after calving, and then for only one milking even though the time from treatment to calving was less than three weeks. Our data seem to contradict the findings of Smith et al. (63) and Ziv et al. (79). However it must be taken into consideration that both of the above

research groups assayed dry udder secretions. In this study samples were taken only from lactating udders. The volume of milk excreted from the lactating cow is extremely large when compared to the 6 ml secretion taken from each quarter of the dry udder as was done by Smith et al. (63) and Ziv et al. (79). Ziv et al. (79) observed that when penicillin G and cloxacillin were infused into the udders of cows producing two to three fold more milk at drying-off than did cows with a lower milk yield, drug levels in the secretions of the former were at times 10 to 100 fold lower than in the secretions of cows with the lower milk yield. In our study, dilution of the cloxacillin and penicillin in the lactating cow may have been the reason for no detectable antibiotic residues in the milk samples.

The antibiotic concentrations listed in Appendix Tables 1 and 2 are approximate values and must not be taken as absolute values. Colostrum is viscous compared to the 2% milk used as diluent for the standard solutions. The colostrum samples did not diffuse into the agar from the cylinders as completely as the 2% milk used in the standards. The cylinder plate method of assay depends on the diffusion of antibiotics into the agar. Thus the cylinder plate test is not well suited for viscous products such as colostrum. Ziv and Rasmussen (77)

indicated that the repeatability of results in the assay of cream was small and in recovery studies low and erratic values were common. The antibiotic concentration observed may well be lower than the actual concentration in the colostrum samples. With the exception of cow 3196, all milk samples from cows that were infused with a dry cow antibiotic preparation during the dry period showed no detectable antibiotic residue beyond the manufacturer's recommended withdrawal time. The manufacturer of the penicillin preparation recommended that the product not be used six weeks prior to the expected date of parturition to insure that the antibiotic would not be excreted longer than the recommended withdrawal time of 96 hours after calving. The manufacturer of the cloxacillin preparation recommended that the product not be used four weeks prior to the expected date of calving to insure that cloxacillin would not be excreted longer than the recommended withdrawal time of 72 hours. However, it should be recommended to dairymen who use dry cow treatment that they dump the milk of treated cows for the specified number of milkings to be sure of complete removal. It should also be recommended that cows that calve up to 10 days after infusion of a dry cow antibiotic preparation have their milk checked for antibiotic residues.

Antibiotic residues in milk following intramuscular
injection and intramammary infusion

The main objective of this study was to determine whether the withdrawal time of penicillin alternately given to lactating cows via intramuscular injection and intramammary infusion would be increased with the use of the Delvotest P over the disc assay and cylinder plate methods of assay. The withdrawal time as defined in this study was the time from the last injection or infusion to the last recorded milk sample taken from any quarter of the treated animal in which penicillin was detected. The mean withdrawal time, the range in time for withdrawal, and the number of milkings for withdrawal as determined with each of the three antibiotic tests used in this study are given in Tables 3 and 4. The mean withdrawal time and range are given in hours. The Delvotest P is divided into two categories; positive (+) and doubtful (+). In the (+) column only definitely positive tests are considered in determining the withdrawal time. (Doubtful tests (+) are considered as negative tests in this column). In the (+) column doubtful tests are considered as positive tests.

Table 3 shows that the Delvotest P detected penicillin residues after intramuscular injection for a longer period

Table 3. Withdrawal times following the final intramuscular injection of 3,000,000 units of procaine penicillin G as determined by the disc assay, cylinder plate, and Delvotest P methods.

Item	Type of antibiotic assay			
	Disc assay	Cylinder plate	Delvotest P	
			+	±
Mean withdrawal				
time (hr)	19.8 ^a	25.0 ^a	33.8 ^b	40.7 ^c
range (hr)	10-34	10-34	24-48	34-58
No. milkings ^d	1-3	1-3	2-4	3-5

^{abc}($P < .01$) Means with the same superscript are not significantly different.

^dManufacturer's recommended withdrawal time is 48 hours.

of time than either the disc assay or cylinder plate methods (33.8 hours versus 19.8 and 25.0 hours respectively). The difference in length of time penicillin was detected was statistically significant ($P < .01$). No significant ($P > .01$) difference in withdrawal time was observed when comparing the disc assay with the cylinder plate method. It is evident that the withdrawal time of penicillin is significantly longer when considering doubtful (+) tests as positive tests than when considering doubtful tests as negative tests.

Table 3 also shows that considerable variation occurred in the withdrawal time of penicillin as determined with each of the three antibiotic tests. A more meaningful representation of the results is made by converting the range for withdrawal time into the number of milkings it would take for complete withdrawal of the penicillin. The manufacturer's recommended withdrawal time for the penicillin preparation used for intramuscular injection was 48 hours or four milkings. If, when using the Delvotest P, doubtful (+) tests are considered as positive tests, the manufacturer's recommended withdrawal time of four milkings would be exceeded by one milking. Since doubtful tests sometimes result when using the Delvotest P to detect antibiotic residues in milk, an interesting question is raised. Should doubtful tests

be considered as positive or as negative tests?

Studies by Marth and Ellickson (38) indicate that a penicillin concentration of .002-.004 units per ml would probably not inhibit the growth of lactic cultures. Doubtful tests occur with the Delvotest P in the range of .002-.004 units of penicillin per ml. Clearly, if a milk sample gave a doubtful test, the milk probably would not inhibit the growth of a lactic culture. However, the minimum concentration of penicillin able to induce hypersensitivity or resistance to penicillin by bacteria is not known. The main reason milk is checked for penicillin residues is that some humans are hypersensitive to penicillin or could become hypersensitive to penicillin (48). Therefore, since penicillin residues in milk in low concentrations can cause doubtful (+) tests, doubtful tests must be considered as positive tests.

Before making a recommendation that the withdrawal time for the penicillin preparation used for intramuscular injection in this study be increased, several factors must be considered. It must be noted (Appendix Tables 3-8) that only milk from the quarter of one cow, cow 3136, gave a doubtful Delvotest P at 58 hours (five milkings). It is very probable that if a composite milk sample from all four quarters was assayed with the Delvotest P, no detectable penicillin residue would have been observed

because of the dilution factor. However, dilution of milk that contains penicillin is illegal even though the assay of diluted milk would show no detectable penicillin residues (48). It should also be taken into account that in no other case was a doubtful test followed by another doubtful test. The doubtful test observed with the milk sample from the right front quarter of cow 3136 may have resulted from other factors besides the presence of penicillin.

Table 4 shows that the Delvotest P did detect penicillin residues after the last intramammary infusion for a longer period of time than did the disc assay method. No significant ($P > .01$) difference in mean withdrawal time was observed in comparing the mean withdrawal time determined with the Delvotest P with that determined with the cylinder plate method. Also, no significant ($P > .01$) difference in mean withdrawal time was noted when comparing the (+) and (+) columns under the Delvotest P.

As with intramuscular injection, considerable variation occurred in the range for withdrawal of penicillin given via intramammary infusion. The manufacturer's recommended withdrawal time for the intramammary penicillin preparation used in this study was 72 hours or six milkings. As can be seen in Table 4, the manufacturer's recommended withdrawal time of six milkings was never

Table 4. Withdrawal times following the final intramammary infusion of 100,000 units of procaine penicillin G as determined by the disc assay, cylinder plate, and Delvotest P methods.

Item	Type of antibiotic assay			
	Disc assay	Cylinder plate	Delvotest P	
			+	±
Mean withdrawal				
time (hr)	35.5 ^a	38.3 ^{ab}	42.7 ^b	43.8 ^b
range (hr)	24-58	24-58	34-72	34-72
No. milkings ^c	2-5	2-5	3-6	3-6

^{ab}($P < .01$) Means with the same superscript are not significantly different.

^cManufacturer's recommended withdrawal time is 72 hours.

exceeded when the milk from cows that had received intramammary infusion was checked with each of the three antibiotic tests.

Crossover of penicillin from the treated quarter (left rear) to the nontreated quarters was never detected. This finding disagrees with the findings of several other studies (1, 10, 20, 26, 34, 52, 54, 60). However, it must be noted that in all these studies reporting penicillin crossover, crossover was not detected in all the treated quarters, or in all cows receiving treatment.

An inverse relationship between milk yield and penicillin concentration is indicated in Tables 5 and 6. As the milk yield increased, the penicillin titer was decreased. The relationship is probably the result of dilution. Since cows were not selected on the basis of production (high and low producers), a comparison of penicillin concentration in milk samples of high and low producers could not be made.

The variation in penicillin concentration in milk from cows that were alternately treated with intramuscular injection and intramammary treatment was analyzed by least squares analysis of variance. The results from analysis of variance of the data are listed in Appendix Table 11. The data show that with intramammary infusion the variation

Table 5. Simple correlation coefficients between milk yield and penicillin titer in milk samples taken from cows after intramuscular treatment.

	Penicillin titer	
	Disc assay	Cylinder plate
Milk yield	-.231**	-.186**

**Significant at 1% level.

Table 6. Simple correlation coefficients between milk yield and penicillin titer in milk samples taken from cows after intramammary treatment.

	Penicillin titer	
	Disc assay	Cylinder plate
Milk yield	-.261**	-.312**

**Significant at 1% level.

in penicillin concentration observed with the disc assay and cylinder plate method is statistically significant ($P < .01$). The variation in penicillin concentration in milk from cows treated with intramuscular injection was not due to the antibiotic assay procedure. The variations in penicillin concentration from separate milkings were also statistically significant for both intramuscular injections and intramammary infusions. However this is to be expected, because as the time from the last injection or infusion increases, the concentration drops.

The penicillin concentrations noted (Appendix Tables 1-10) must not be taken as absolute values. The limit of the penicillin standard used for the disc assay was .025 units of penicillin per ml of sample. The limit of the penicillin standard used for the cylinder plate antibiotic test was .0125 units of penicillin per ml of sample. Penicillin concentrations (Appendix Tables 1-10) below the limits of the standards must be considered as extrapolated values.

A comparison of the relative sensitivity of the disc assay, cylinder plate, and Delvotest P methods for detection of penicillin in raw milk is shown in Table 7. The total number of positive tests for the presence of penicillin as detected by the Delvotest P was 588. The disc assay antibiotic test detected penicillin in 75% of the milk

Table 7. Comparison of the relative sensitivity of the disc assay, cylinder plate, and Delvotest P methods for detecting penicillin in raw milk.

Type of treatment	Type of antibiotic assay			
	Disc assay	Cylinder plate	Delvotest P	
			+	-
Number of positive tests.				
Intramuscular injection	359	402	469	29
Intramammary infusion	82	85	89	1
TOTAL	441	487	558	30
Sensitivity as % of Delvotest P	75	83	95	5

samples in which penicillin was detected by the Delvotest P method. The cylinder plate method detected penicillin in 83% of the milk samples in which penicillin was detected by the Delvotest P method. The results of our study agree with the study by Packard et al. (43).

The results of the comparison of the relative sensitivity of the three antibiotic tests used in this study show that a larger number of positive identifications of penicillin adulterated milk would be anticipated if the Delvotest P were used to assay milk samples instead of the disc assay or cylinder plate methods. In addition, an assay with the Delvotest P is simpler and faster to complete than either the disc assay or cylinder plate tests. Also, the cylinder plate method is more sensitive to penicillin residues in milk than the disc assay. However, for routine laboratory analysis of a large number of samples the disc assay would be preferred over the cumbersome cylinder plate method.

SUMMARY

Milk samples from six cows that alternately received intramuscular injections and intramammary infusions of penicillin were analyzed for the presence of penicillin residues using the disc assay, cylinder plate, and Delvotest P methods. Withdrawal times for milk from the treated cows were determined with each of the three tests.

The disc assay antibiotic test detected penicillin in only 75% of the milk samples in which penicillin was detected by the Delvotest P. The cylinder plate method detected penicillin in only 83% of the milk samples in which penicillin was detected by the Delvotest P method. Also, the Delvotest P method detected penicillin residues for a significantly longer ($P < .01$) time than either the disc assay or cylinder plate methods in milk from cows following intramuscular injections. In one case, the manufacturer's recommended withdrawal time of four milkings was exceeded, but then by only one milking.

In milk from cows receiving intramammary infusions, the Delvotest P detected penicillin residues for a significantly ($P < .01$) longer period than the disc assay method, but not longer than the cylinder plate method. The manufacturer's recommended withdrawal time of six milkings was never exceeded as measured by any of the three

antibiotic tests. No detectable penicillin crossover from treated to untreated quarters was observed.

Nineteen cows were treated via intramammary infusion at various times prior to calving with one of two dry cow antibiotic preparations (cloxacillin or penicillin). Milk samples taken from these cows after calving were analyzed for antibiotic residues using the Sarcina lutea cylinder plate method. Eight cows were treated with cloxacillin. However, cloxacillin was detected in the milk of only two cows after calving and then for only one milking.

Penicillin residues were not detected in milk samples taken from the two cows treated more than nine days prior to calving with the dry cow penicillin preparation. Seven out of nine cows treated with penicillin up to nine days prior to calving had detectable penicillin residues in the milk from treated quarters after calving. However, only one cow had detectable penicillin residues longer than the manufacturer's recommended withdrawal time of eight milkings, and this cow was treated only three days prior to parturition.

APPENDIX

Appendix Table 1. Dry cow treatment—cloxacillin concentrations in milk from cows given 500 mg of benzathine cloxacillin at various times prior to calving.

Cow Number	Interval to calving after treatment	Treated quarters ^a	Hours after calving						
			0	12	24	36	48	60	72
Days			(µg/ml) ^b						
3238	8	Left front	---	.830	0	0	0	0	
		Right rear	---	1.65	0	.0	0	0	
3307	8	Left front	.165	0	0	0			
		Left rear	0	0	0	0			
3021	14	Left front	---	0	0	0			
		Right rear	---	0	0	0			
3176	11	Right front	0	0	0	0			
		Left front	0	0	0	0			
3031	10	Left front	---	---	---	0	0	0	0
		Left right	---	---	---	0	0	0	0
3030	7	Right front	---	0	0	0			
		Right rear	---	0	0	0			
3167	18	Left front	---	0	0	0			
		Right rear	---	0	0	0			
3276	9	Right front	---	0	0	0			
		Right rear	---	0	0	0			

^aNo detectable antibiotic residues were present in samples from nontreated quarters.

^bZero indicates no detectable antibiotic residue, (---) = sample not collected.

Manufacturer's recommended withdrawal time is 72 hours after calving.

Appendix Table 2. Dry cow treatment—penicillin concentrations in milk from cows given 1,000,000 units of procaine penicillin G at various times prior to calving.

Cow Number	Interval to calving after treatment	Treated quarters ^a	Hours after calving										
			0	12	24	36	48	60	72	84	96	108	120
			(units/ml) ^d										
—Days—													
3186	3	LR	----	15.6	23.8	.255	.0400	.0519	.0249	.114	.00940	.0141	0
		IF	----	22.4	2.56	.464	.122	.170	.0421	0	.0161	0	0
3315	1	LR ^b	.0325	0	0								
		IF	.93.0	5.25	4.23	1.11	.272	.0522	0	0			
		RF ^b	.0213	0	0								
		RR	.54.4	4.75	.355	.110	.300	.0112	.0205	0	0		
3273	7	LR	.0235	----	.195	.0580	.0330	.0370	.0130	0	0		
		RR	0	0	0	0	0	0	0				
3293	5	LR	----	.392	.329	.113	.00274	0	0	0	0		
		RF	----	.686	.210	.0748	.0273	.0238	0	0	0		
3024	9	IF	----	.0188	0	0	0	0					
		RR	----	1.32	.807	.271	.115	0	0				
3147	5	LR	.110	.0213	0	0							
		IF	.393	.0407	0	0							
		RF ^b	0	0	NP	0							
		RR ^b	0	NP	0	0							
3315	4	LR ^b	1.70	.500	.437	.0383	.0491	.00901	0	0			
		IF ^b	0	0	0	.0183	0	0	0				
		RF	.365	0	0	.0233	.0334	0	0				
		RR ^{bc}	0	0	0	105	27.0	25.1	2.59	.0134	0	0	

Table continued on next page.

Appendix Table 2, continued.

Cow Number	Interval to calving- after treatment	Treated quarters ^a	Hours after calving									
			0	12	24	36	48	60	72	84	96	108
—Days—			(units/ml) ^d									
3023	9	RF	0	0	0	0						
		RR	0	0	0	0						
3029	10	LF	----	0	0	0	0	0				
		LR	----	0	0	0	0	0				
3231	8	RF	0	0	0							
		RR	0	0	0							
3152	24	LF	0	0	0							
		LR	0	0	0							

^aNo detectable antibiotic residues were present in samples from nontreated quarters, LF = left front, LR = left rear, RF = right front, RR = right rear.

^bQuarters were not treated.

^cRight rear quarter treated for an infection.

^dZero indicates no detectable antibiotic residue, RF = sample gave positive test but was not due to penicillin, (----) = sample not collected.

Manufacturer's recommended withdrawal time is 96 hours after calving.

Appendix Table 3. Procaine penicillin G in milk after intramuscular injection.^a

Cow number 2845.

Quarter	Type of assay ^b	Hours after first injection		Hours after second injection		Hours after third injection		Hours after fourth injection		Hours after fifth injection				
		10	24	10	24	10	24	10	24	10	24	34	48	56
Rep. 1, set 1		(units/ml) ^c												
Left rear	DA	.0423	0	.0452	0	.0551	0	.0467	.0191	.0383	0	0		
	CP	.0409	.0165	.0582	.00663	.0403	0	.0384	.00746	.0333	.0288	0		
	Del-P		+		+		+		+		+	±	-	-
Left front	DA	.0266	0	.0370	0	.0483	0	.0437	0	.0423	0	0		
	CP	.0241	0	.0621	0	.0452	.00664	.0390	.00807	.0328	.00934	0		
	Del-P		-		+		+		+		+	+	-	-
Right front	DA	.0266	.0162	.0396	.0162	.0516	0	.0437	0	.0409	.0191	0		
	CP	.0267	.0201	.0582	.0139	.0486	.00742	.0333	.00847	.0367	.0160	0		
	Del-P		+		+		+		+		+	+	-	-
Right rear	DA	.0284	.0167	.0294	0	.0396	0	.0266	.0137	.0423	.0162	0		
	CP	.0294	.0201	.0545	.00842	.0349	.00958	.0262	.0132	.0333	.0179	0		
	Del-P		+		+		+		+		+	+	-	-
Rep. 2, set 1														
Left rear	DA	.0298	.0148	.0357	0	.0824	.0178	.0494	0	.0366	0			
	CP	.0369	.0270	.0468	.00852	.0435	.0211	.0432	.00992	.0304	0	0		
	Del-P										+	+	-	-
Left front	DA	.0323	.0113	.0248	.0107	.0364	.0145	.0351	.0102	.0143	0			
	CP	.0449	.0162	.0364	.0125	.0449	.0215	.0358	.0120	.0117	.0114	0		
	Del-P										+	+	-	-
Right front	DA	.0298	.0148	.0378	0	.0328	.0166	.0311	.00742	.0298	.0150	0		
	CP	.0334	.0201	.0358	.00926	.0328	.0228	.0278	.0163	.0277	.0166	0		
	Del-P										+	+	-	-
Right rear	DA	.0282	.0148	.0319	0	.0236	.0178	.0296	.0127	.0231	0			
	CP	.0320	.0284	.0402	0	.0396	.0259	.0352	.0130	.0237	.00834			
	Del-P				±						+	+	±	-

^a Each injection consisted of 3,000,000 units of procaine penicillin G in aqueous suspension. (Treatment schedules described in Table 2, Materials and Methods section.)

^b DA = Disc assay, CP = Cylinder plate, Del-P = Delvotest P.

^c zero indicates no detectable antibiotic residue; (+) = positive test, (-) = negative test, (±) = doubtful test.

Appendix Table 4. Procaine penicillin G in milk after intramuscular injection.^a

Cow number 2955.

Quarter	Type of assay ^b	Hours after first injection		Hours after second injection		Hours after third injection		Hours after fourth injection		Hours after fifth injection				
		10	24	10	24	10	24	10	24	10	24	34	48	53
Rep. 1, set 2		(units/ml) ^c												
Left rear	DA	.0287	.0156	.0353	.0126	.0379	.0277	.0557	0	.0348	0			
	CP	.0241	.0162	.0366	.0154	.0321	.0127	.0615	.00712	.0277	.00963	0		
	Del-P								+		+	±	-	-
Left front	DA	.0412	0	.0456	.0186	.0520	.0314	.0682	0	.0422	0			
	CP	.0306	.0109	.0334	.0154	.0425	.0101	.0566	0	.0292	.0126	0		
	Del-P								+		+	±	-	-
Right front	DA	.0243	.0264	.0192	.0148	.0488	.0345	.0321	0	.0358	0			
	CP	.0274	.0165	.0169	.0185	.0440	.0418	.0289	.0123	.0259	.0130	0		
	Del-P								+		+	+	-	-
Right rear	DA	.0389	.0156	.0427	.0118	.0758	.0222	.0321	0	.0339	0			
	CP	.0343	.0212	.0349	.0138	.0405	.0127	.0547	0	.0250	.0130	0		
	Del-P								+		+	±	-	-
Rep. 2, set 1														
Left rear	DA	.0464	.0146	.0440	.0163	.0259	.0117	.0366	.0234	.0393	.0214	0	0	
	CP	.0375	.0127	.0345	.0114	.0231	.0110	.0413	.0259	.0378	.0196	.0174	0	
	Del-P											+	-	-
Left front	DA	.0405	.0142	.0416	.0138	.0527	0	.0453	0	.0404	.0208	0	0	
	CP	.0469	.0125	.0340	.0175	.0348	0	.0432	0	.0378	.0212	.0123	0	
	Del-P						+		±			+	-	-
Right front	DA	.0464	.0167	.0428	.0214	.0282	.0252	.0297	.0274	.0290	.0232	.0151	0	
	CP	.0404	.0149	.0357	.0145	.0327	.0207	.0347	.0313	.0309	.0256	.0128	0	
	Del-P											+	±	-
Right rear	DA	.0519	.0124	.0372	.0182	.0259	0	.0441	.0247	.0306	.0181	0	0	
	CP	.0423	.0151	.0370	.0138	.0231	0	.0396	.0193	.0390	.0222	.0143		
	Del-P						+					+	±	-

^a Each injection consisted of 3,000,000 units of procaine penicillin G in aqueous suspension. (Treatment schedules described in Table 2, Materials and Methods section.)

^b DA = Disc assay, CP = Cylinder plate, Del-P = Delvotest P.

^c Zero indicates no detectable antibiotic residue; (+) = positive test, (-) = negative test, (±) = doubtful test.

Appendix Table 5. Procaine penicillin G in milk after intramuscular injection.^a

Cow number 3237.

Quarter	Type of assay ^b	Hours after first injection		Hours after second injection		Hours after third injection		Hours after fourth injection		Hours after fifth injection				
		10	24	10	24	10	24	10	24	10	24	34	48	52
Rep. 1, set 1		-(units/ml) ^c												
Left rear	DA	.0672	.0396	.0767	.0718	.0672	.0249	.139	.0191	.0467	0			
	CP	.0591	.0360	.0465	.0354	.0922	.0325	.0931	.0223	.0370	0	0		
	Del-P									+	+	±	-	-
Left front	DA	.194	.0718	.181	.0483	.0629	.0396	.207	.0226	.0314	0			
	CP	.0746	.0582	.0390	.0129	.0922	.0377	.103	.0253	.0296	.00756	0		
	Del-P									+	+	±	-	-
Right front	DA	.149	.0191	.0967	.0876	.0767	.0266	.164	.0266	.0275	0			
	CP	.0769	.0256	.0591	.0416	.0993	.0357	.0906	.0214	.0274	0	0		
	Del-P									+	+	±	-	-
Right rear	DA	0	.0516	.139	.0718	.0763	.0304	.170	.0198	.0370	0			
	CP	0	.0338	.0581	.0429	.0957	.0384	.0799	.0229	.0365	0	0		
	Del-P	-								+	+	±	-	-
Rep. 2, set 1														
Left rear	DA	.0323	0	.0373	.0266	.0201	0	0	0	.0350	.00973	0	0	
	CP	.0271	0	.0231	.0269	.0110	0	0	0	.0233	.0172	.0103	0	
	Del-P		+			+	+	-	-	+	+	+	+	-
Left front	DA	.0370	.0209	.0342	.0333	.0191	0	0	0	.0294	.0175	0	0	
	CP	.0238	.0270	.0231	.0243	.0138	0	0	0	.0531	.0188	.00916	0	
	Del-P					+	+	±	-	+	+	+	-	-
Right front	DA	.0360	.0267	.0508	.0224	0	0	0	0	.0248	.0164	0	0	
	CP	.0336	.0266	.0315	.0337	0	0	0	0	.0416	.0248	0	0	
	Del-P					+	+	-	-	+	+	+	+	-
Right rear	DA	.0260	.0227	.0306	.0417	0	0	0	0	.0275	.0164	0	0	
	CP	.0245	.0298	.0343	.0284	0	0	0	0	.0346	.0233	.00901	0	
	Del-P					+	+	±	-	+	+	+	+	-

^a Each injection consisted of 3,000,000 units of procaine penicillin G in aqueous suspension. (Treatment schedules described in Table 2, Materials and Methods section.)

^b DA = Disc assay, CP = Cylinder plate, Del-P = Delvotest P.

^c Zero indicates no detectable antibiotic residue; (+) = positive test, (-) = negative test, (±) = doubtful test.

Appendix Table 6. Procaine penicillin G in milk after intramuscular injection.^a

Cow number 3136.

Quarter	Type of assay ^b	Hours after first injection		Hours after second injection		Hours after third injection		Hours after fourth injection		Hours after fifth injection				
		10	24	10	24	10	24	10	24	10	24	34	48	58
Rep. 2, set 2		(units/ml) ^c												
Left rear	DA	.0532	.0140	.0470	.0304	.0460	0	.0158	0	.0659	.0201	0	0	
	CP	.0451	.0138	.0450	.0269	.0364	0	.0199	0	.0645	.0144	0	0	
	Del-P						+		+			+	±	-
Left front	DA	.0506	.0214	.0498	.0234	.0424	0	.0201	0	.0659	.0237	0	0	
	CP	.0386	.0224	.0421	.0161	.0261	0	0	0	.0656	.0220	.0101	0	
	Del-P						-	+	+			+	±	-
Right front	DA	.0413	.0214	.0341	.0304	.0401	.0141	.0206	.0158	.0941	.0294	0	0	
	CP	.0438	.0155	.0305	.0199	.0225	0	.0199	0	.0701	.0295	0	0	
	Del-P						+		+			+	±	±
Right rear	DA	.0355	.0214	.0248	.0270	.0379	0	.0158	.0178	.0691	.0419	.0267	0	
	CP	.0325	.0134	.0241	.0193	.0273	0	0	0	.0594	.0319	.0142	0	
	Del-P						-	+	+			+	±	-
Rep. 1, set 2		(10 24 34 48 58) ^d												
Left rear	DA	0	0	.0659	0	.0280	0	.0497	0	0	0			
	CP	0	.00897	.0558	.00874	.0284	.0108	.0318	.0138	0	0			
	Del-P	+	+		+				+	±	-	-		
Left front	DA	.0299	.0211	.0570	0	.0119	.0244	.0379	.0177	0	0			
	CP	.0324	.0191	.0540	.00770	.0297	.0173	.0220	.0152	0	0			
	Del-P				+				+	±	-	-		
Right rear	DA	.0194	.0123	.0718	0	.0543	0	.0511	0	0	0			
	CP	.0243	.0185	.0664	.00758	.0401	0	.0483	.0126	0	0			
	Del-P						+			±	-	-		
Right front	DA	.0126	.0234	.0534	.0132	.0322	.0225	.0289	0	0	0			
	CP	.0109	.0251	.0507	.0138	.0263	.0210	.00822	.0159	0	0			
	Del-P	+								+	-	-		

^aEach injection consisted of 3,000,000 units of procaine penicillin G in aqueous suspension. (Treatment schedules described in Table 2, Materials and Methods section.)

^bDA = Disc assay, CP = Cylinder plate, Del-P = Delvotest P.

^cZero indicates no detectable antibiotic residue; (+) = positive test, (-) = negative test, (±) = doubtful test.

^dCow given only four injections. Hours listed represent hours after fourth injection.

Appendix Table 7. Procaine penicillin G in milk after intramuscular injection.^a

Cow number 3092.

Quarter	Type of assay ^b	Hours after first injection		Hours after second injection		Hours after third injection		Hours after fourth injection				
		10	24	10	24	10	24	10	24	34	48	50
Rep. 2, set 2 ^d		(units/ml) ^c										
Left rear	DA	.0795	.0223	.133	.0147	.140	0	.0938	0			
	CP	.0558	.0186	.0995	.0114	.103	0	.112	0	0	0	
	Del-P						+		+	-	-	-
Left front	DA	.0613	.0338	.0953	.0143	.136	0	.0962	0			
	CP	.0449	.0189	.103	.0145	.0807	0	.0997	0	0	0	
	Del-P						+		+	-	-	-
Right front	DA	.0365	.0338	.117	.0147	.101	0	.0849	0			
	CP	.0350	.0227	.0858	.0143	.0603	0	.0940	0	0	0	
	Del-P						+		+	±	-	-
Right rear	DA	.0438	.0212	.0384	.0212	.0871	0	.0731	0			
	CP	.0373	.0278	.0492	.0272	.111	0	.0968	0	0	0	
	Del-P						+		+	±	-	-
Rep. 1, set 1		(10 24 34 48 58 72) ^e										
Left rear	DA	.120	.0326	.0467	.0145	.0816	.0204	0	0			
	CP	.107	.0513	.0507	.0159	.0935	.0207	.00480	0			
	Del-P							+	+	-	-	
Left front	DA	.106	.0268	.0540	.0168	.0778	0	0	0			
	CP	.131	.0489	.0631	.0204	.0782	.00635	.00498	0			
	Del-P						+	+	-	-	-	
Right front	DA	.106	.0371	.0316	0	.0816	.0143	0	0			
	CP	.121	.0489	.0531	.0144	.0782	.0127	.00616	0			
	Del-P							+	+	-	-	
Right rear	DA	.117	.0384	.0349	.0209	.0778	.0209	0	0			
	CP	.107	.0555	.0574	.0271	.0964	.0165	0	0			
	Del-P							+	+	-	-	

^a Each injection consisted of 3,000,000 units of procaine penicillin G in aqueous suspension. (Treatment schedules described in Table 2, Materials and Methods section.)

^b DA = Disc assay, CP = Cylinder plate, Del-P = Delvotest P.

^c Zero indicates no detectable antibiotic residue; (+) = positive test, (-) = negative test, (±) = doubtful test.

^d Cow given only four injections.

^e Cow given only three injections. Hours listed represent hours after third injection.

Appendix Table 8. Procaine penicillin G in milk after intramuscular injection.^a Cow number 3167.

Quarter	Type of assay ^b	Hours after first injection		Hours after second injection		Hours after third injection		Hours after fourth injection		Hours after fifth injection				
		10	24	10	24	10	24	10	24	10	24	34	48	53
Rep. 1, set 2		(units/ml) ^c												
Left rear	DA	.0514	0	.0316	.0262	.0521	.0254	.0509	.0271	.0204	0			
	CP	.0457	0	.0329	.0173	.0467	.0211	.0458	.0243	.0151	.00815	0	0	
	Del-P		+								+	+	-	-
Left front	DA	.0672	0	.0365	.0302	.0410	.0228	.0551	.0334	.0242	0			
	CP	.0398	0	.0318	.0185	.0433	.0162	.0382	.0228	.0168	.00865	0	0	
	Del-P		-								+	+	-	-
Right front	DA	.0543	0	.0365	0	.0662	.0254	.0523	.0250	.0247	0			
	CP	.0333	.0112	.0255	.0111	.0561	.0162	.0450	.0161	.0189	.00827	0	0	
	Del-P		+								+	+	-	-
Right rear	DA	.0488	0	.0420	.0268	.0306	.0247	.0496	.0264	.0253	0			
	CP	.0328	0	.0335	.0132	.0396	.0170	.0340	.0141	.0189	0	0		
	Del-P		+							+	+	+	-	-
Rep. 2, set 2														
Left rear	DA	.0431	.0160	.0583	.0285	.0816	.0290	.0511	.0298	.0212	0			
	CP	.0494	.0170	.0404	.0137	.0458	.0341	.0391	.0245	.0137	0			
	Del-P									+	+	±	-	-
Left front	DA	.0386	.0152	.0468	.0302	.0646	.0376	.0598	.0290	0	0			
	CP	.0592	.0150	.0237	.0165	.0555	.0313	.0373	.0203	.0126	0			
	Del-P									+	+	±	-	-
Right front	DA	.0397	.0200	.0669	.0242	.0663	.0462	.0406	.0276	.0148	0			
	CP	.0350	.0173	.0359	.0170	.0565	.0324	.0416	.0241	.0126	0			
	Del-P									+	+	±	-	-
Right rear	DA	.0494	.0160	.0386	0	.0499	.0366	.0439	.0255	.0172	0			
	CP	.0485	.0150	.0280	0	.0379	.0237	.0248	.0233	.0135	0			
	Del-P				±					+	+	±	-	-

^a Each injection consisted of 3,000,000 units of procaine penicillin G in aqueous suspension. (Treatment schedule described in Table 2, Materials and Methods section.)

^b DA = Disc assay, CP = Cylinder plate, Del-P = Delvotest P.

^c Zero indicates no detectable antibiotic residue; (+) = positive test, (-) = negative test, (±) = doubtful test.

Appendix Table 9. Procaine penicillin G in milk after intramammary infusion.^a

Cow Number	Infused Quarter ^b	Type of assay ^c	Hours after first infusion		Hours after second infusion		Hours after third infusion					
			10	24	10	24	10	24	34	48	58	72
—————(units/ml) ^d —————												
2845	Left rear	DA	11.5	.398	11.4	.293	25.2	.394	.150	0	0	
		CP	11.8	.552	12.6	.234	31.9	.360	.135	0	0	
		Del-P							+	-	-	

Rep. 1, set 2	Left rear	DA	17.5	.293	12.2	.746	34.7	1.00	.130	0		
		CP	21.0	.277	17.3	.526	37.4	1.02	.115	0		
		Del-P							+	+	-	-

2955	Left rear	DA	1.32	.214	12.2	1.01	59.6	.831	.0347	0		
		CP	6.13	.488	12.8	.940	44.2	.285	.0649	0		
		Del-P							+	-	-	-

Rep. 1, set 1	Left rear	DA	27.4	.734	13.0	.771	20.6	.758	.0330	0		
		CP	25.6	.786	17.3	.689	23.2	.634	.0259	0	0	
		Del-P							+	-	-	

Rep. 2, set 2	Left rear	DA	27.4	.734	13.0	.771	20.6	.758	.0330	0		
		CP	25.6	.786	17.3	.689	23.2	.634	.0259	0	0	
		Del-P							+	-	-	

3136	Left rear	DA	32.8	.0767	6.94	.0570	6.94	.288	.170	0	0	
		CP	24.2	.0332	7.20	.0591	10.5	.243	.0906	.00650	0	
		Del-P							+	+	-	-

Rep. 1, set 1	Left rear	DA	11.9	.798	8.77	.201	7.09	.0348	.0691	0		
		CP	9.88	.562	8.82	.163	7.24	.0349	.0171	0		
		Del-P							+	-	-	

Rep. 2, set 1	Left rear	DA										
		CP										
		Del-P										

^aEach infusion consisted of 100,000 units of procaine penicillin G in an ointment based preparation. (Treatment schedules described in Table 2, Materials and Methods section.)

^bNo detectable crossover occurred between treated and nontreated quarters.

^cDA = Disc assay, CP = Cylinder plate, Del-P = Delvotest P.

^dZero indicates no detectable antibiotic residue; (+) = positive test, (-) = negative test.

Appendix Table 10. Procaine penicillin G in milk after intramammary infusion.^a

Cow Number	Infused Quarter ^b	Type of assay ^c	Hours after first infusion		Hours after second infusion		Hours after third infusion							
			10	24	10	24	10	24	34	48	58	72	82	
(units/ml) ^d														
3167	Left rear	DA	7.87	.720	5.80	.512	LA	1.85	.293	.0726	.0106	0	0	
		CP	9.46	.652	LA	.653	LA	.346	.218	.0366	.00714	0	0	
Rep. 1, set 1		Del-P								+	+	+	-	
	Left rear	DA	18.6	1.34	22.2	1.83	23.2	1.74	.596	.0974	0	0		
		CP	18.2	1.97	23.8	2.44	23.4	1.94	.578	.104	.0106	0	0	
Rep. 2, set 1		Del-P								+	+	+	-	
3092	Left rear	DA	15.9	.125	10.1	.131	46.4	.508	.0209	0				
		CP	12.3	.0230	10.8	.118	55.0	.403	.0122	0	0	-	-	
Rep. 1, set 2		Del-P							+	+				
	Left rear	DA	11.0	.116	12.1	.219	9.56	.206	0	0				
		CP	8.92	.125	8.38	.252	8.26	.237	.0104	0	0	-	-	
Rep. 2, set 1		Del-P							+	-	-	-		
3287	Left rear	DA	20.3	.0436	22.9	.257	20.9	.102	.0159	0				
		CP	15.2	.0394	21.6	.267	22.0	.184	.00971	0				
Rep. 1, set 2		Del-P							+	-	-			
(10 24 34 48 58) ^e														
	Left rear	DA	10.3	.304	10.9	.305	0	0						
		CP	11.4	.302	13.1	.434	0	0						
Rep. 2, set 2		Del-P				+	+	-	-					

^a Each infusion consisted of 100,000 units of procaine penicillin G in an ointment based preparation. (Treatment schedules described in Table 2, Materials and Methods section.)

^b No detectable crossover occurred between treated and non-treated quarters.

^c DA = Disc assay, CP = Cylinder plate, Del-P = Delvotest P.

^d Zero indicates no detectable antibiotic residue; (+) = positive test, (-) = negative test, (±) = doubtful test, (LA) = laboratory accident.

^e Cow given only two infusions. Hours listed represent hours after second infusion.

Appendix Table 11. Analysis of variance for penicillin concentration variations due to various factors following intramuscular injection and intramammary infusion.^a

Source of variation	Degrees of freedom	Mean square	
		Intramuscular injection	Intramammary infusion
Rep	1	.0011	136.89
Set/rep	2	.0021	128.66
Cow/SR	8	.0026	207.88
Milkings	2	.0435 **	2761.99 **
MxR	2	.000060	38.62
MxS/R	4	.0032	92.69
MxC/SR	16	.0016	331.83
Test	1	.000030	16.11 **
RxT	1	.00015	.093
TxS/R	2	.00016	26.88
TxC/SR	8	.00010	10.26
TxM	2	.00021	4.29
TxMxR	2	.00025	15.14
TxMxS/R	4	.00010	6.69
TxMxC/SR	16	.000060	14.38
Quarters	3	.000040	
QxR	3	.000020	
QxS/R	6	.000064	
QxC/SR	24	.000060	
QxM	6	.00010	
QxMxR	6	.000030	
QxMxS/R	12	.000040	
QxMxC/SR	48	.000050	
QxT	3	.000070	
QxRxT	3	.000030	
QxTxS/R	6	.000020	
QxTxC/SR	24	.000040	
QxTxM	6	.000015	
QxTxMxR	6	.000030	
QxTxMxS/R	12	.000020	
QxTxMxC/SR	48	.000020	

^a Only the penicillin concentration detected in milk samples from the last injection or infusion was used.

**Significant ($P < .01$).

Appendix Table 12. Analysis of variance for variations in mean withdrawal time of penicillin due to various factors following intramuscular injection and intramammary infusion.

Source of variation	Degrees of freedom	Mean square	
		Intramuscular injection	Intramammary infusion
Cow	5	129.13	980.93**
Rep/cow	6	304.83	91.83
Test ^a	3	1026.89**	179.22**
CxT	15	16.55	20.09
TxR/cow	18	30.39	24.00

^aTests used were disc assay, cylinder plate, and Delvotest P. (Delvotest P taken as two separate tests; one test used only positive (+) tests for determination of withdrawal time. The other test used doubtful (+) tests for determination of withdrawal time).

**Significant ($P < .01$).

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